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Detection of Airborne Salmonella enteritidis in the Environment of Experimentally Infected Laying Hens by an Electrostatic Sampling Device

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SUMMARY. Bacteriologic culturing of environmental samples taken from sources such as manure pits and egg belts has been the principal screening tool in programs for identifying commercial laying flocks that have been exposed to *Salmonella enteritidis* and are thus at risk to produce contaminated eggs. Because airborne dust and aerosols can carry bacteria, air sampling offers a potentially efficient and inexpensive alternative for detecting *S. enteritidis* in poultry house environments. In the present study, an electrostatic air sampling device was applied to detect *S. enteritidis* in a room containing experimentally infected, caged laying hens. After oral inoculation of hens with a phage type 13a *S. enteritidis* strain, air samples were collected onto agar plates with the electrostatic sampling device, an impaction air sampler, and by passive exposure to the settling of aerosols and dust. Even though the floor of the room was cleaned once per week (removing most manure, dust, and feathers), air samples were positive for *S. enteritidis* for up to 4 wk postinoculation. On the basis of both the number of *S. enteritidis* colonies observed on incubated agar plates and the frequency of positive results, the efficiency of the electrostatic device was significantly greater than that of the passive exposure plates (especially at short collection intervals) and was similar to that of the far more expensive impaction sampler. The electrostatic device, used for a 3-hr sampling interval, detected airborne *S. enteritidis* on 75% of agar plates over the 4 wk of the study.

RESUMEN. Detección de Salmonella enteritidis en el aire ambiental de gallinas de postura infectadas experimentalmente, mediante el uso de un aparato de muestreo electrostático.

Los cultivos bacteriológicos de muestras de ambiente obtenidas de fosas de excretas y bandas transportadoras de huevo han sido los métodos principales de muestreo en programas para identificar parvadas de postura comerciales que han sido expuestas a *Salmonella enteritidis* y por lo tanto representan un riesgo de producir huevos contaminados. Debido a que el polvo y los aerosoles pueden transportar bacterias, la realización de muestreos en el aire puede ser una alternativa eficiente y accesible para detectar *S. enteritidis* en el ambiente de las casetas avícolas. En el presente estudio se utilizó un aparato electrostático para el muestreo de aire con el fin de detectar *S. enteritidis* en un alojamiento que contenía gallinas de postura enjauladas que fueron infectadas experimentalmente. Después de la inoculación oral de las gallinas con *S. enteritidis* fagotipo 13a, se tomaron muestras de aire en placas de agar mediante el aparato electrostático de muestreo. También se realizaron muestreos con aparatos de muestreo por impactación de aire y mediante exposición pasiva a aerosoles y polvo. A pesar de que el piso del alojamiento se limpiaba una vez por semana (removiendo excretas, polvo y plumas), las muestras de aire fueron positivas a *S. enteritidis* hasta cuatro semanas después de la inoculación. Con base al número de colonias observadas en las placas de agar y a la frecuencia de resultados positivos, se determinó que la eficiencia del aparato electrostático de muestreo fue significativamente mayor en comparación con la exposición pasiva de las placas de agar, (especialmente en intervalos cortos) y fue similar al aparato de muestreo por impactación que es más costoso. El aparato electrostático de muestreo usado durante intervalos de tres horas detectó *S. enteritidis* en el aire en 75% de las placas de agar durante las cuatro semanas del estudio.

Key words: *Salmonella enteritidis*, chickens, egg contamination, air, electrostatic sampling device

Abbreviations: BG = brilliant green; ELISA = enzyme-linked immunosorbent assay; MAC = MacConkey; PI = postinoculation

Contamination of the edible contents of table eggs with *Salmonella enteritidis* is an internationally significant cause of human illness (1,5). A recent survey reported a 7.1% prevalence of *S. enteritidis* in the housing environments of commercial egg-laying flocks in the United States (9). Because systemically infected hens can deposit *S. enteritidis* inside eggs before oviposition (10,12,13), identification of infected flocks is a principal objective in efforts to reduce egg-associated transmission of disease to consumers (23). Typical of such programs, a proposed national testing program for *S. enteritidis* in laying flocks in the United States would employ a two-step approach (34). An initial screening step involving bacteriologic culturing of samples from the laying house environment would be followed by culturing of eggs from environmentally positive flocks to provide conclusive evidence of the need for regulatory intervention (such as the diversion of eggs for pasteurization). Used in conjunction with a comprehensive risk reduction program, a similar testing plan was associated with a significant reduction in the environmental prevalence of *S. enteritidis* in Pennsylvania laying flocks (39).

Although the detection of *S. enteritidis* in the environment of laying houses provides a sensitive and meaningful indicator of flock infection (22), collection and processing of environmental samples can be labor intensive and time consuming. Accordingly, alternative choices for this initial screening step remain of interest. Tests for specific antibodies are attractive because they are rapid and easily automated. Flock testing programs based on preliminary serologic screening have been reported effective in both the United States and Europe (35,41). However, the widespread application of serologic assays is hindered by lingering concerns about the specificity of antibody detection (17,18). Tests to detect airborne bacteria are attractive because a relatively small number of easily collected samples can provide information about the presence of pathogens circulating throughout a poultry house. However, most efficient air sampling devices are prohibitively expensive for routine use.

Air circulation is often an important factor in the transmission and perpetuation of *Salmonella* infection in poultry flocks. Chickens can be readily infected with *S. enteritidis* by exposure to contaminated aerosols (3). Airborne horizontal transmission of *S. enteritidis* infection has been observed in both chicks and laying hens in a variety of housing situations (15,24,30,33). Air movement within poultry houses also influences the environmental persistence of salmonellae. For example, moist litter in areas of a broiler house with low airflow can support the survival of high bacterial levels (8,31). Extended environmental persistence of *S. enteritidis* on poultry farms (6,21) might allow widespread airborne dissemination of the pathogen via contaminated dust and aerosols. This airborne circulation of bacteria may offer an opportunity to efficiently identify infected flocks by collecting and testing air samples. Air samples have been used to detect *Salmonella* and other bacteria in poultry hatchers, houses, and processing facilities (2,27,40). Both passive exposure of agar plates and devices that pull air either over agar surfaces (impaction) or into liquid media (impingement) have been reported effective in detecting airborne salmonellae (4,27). Air filters from poultry housing ventilation systems have also been used successfully as samples for detecting the presence of *Salmonella* (28,29).

Electrostatic space chargers (negative air ionizers) have been found to reduce dust levels in poultry hatchers and housing facilities (16,32,36), thereby also diminishing airborne bacterial levels (25,36) and the experimental transmission of *Salmonella* to chicks (16,32). Negative air ionization can also reduce *S. enteritidis* numbers in aerosols and on exposed surfaces (37). The ability of electrostatic charging to attract and hold bacteria borne on airborne particulate matter is critical to the success of this technology as a control strategy for reducing the environmental levels of pathogens. This same attribute might also be adaptable as a technique for detecting the presence of airborne pathogens. The objective of the present study was to evaluate the effectiveness of an electrostatic sampling device for detecting the presence of airborne *S. enteritidis* in a room containing experimentally infected laying hens.

MATERIALS AND METHODS

Experimental infection of laying hens. In each of two replicate trials, 36 laying hens from our laboratory's specific-pathogen-free flock of single-comb white leghorn chickens were housed individually in laying cages in a disease-containment facility. The hens

(39 wk old at the beginning of each trial) were distributed evenly throughout two tiers of cages located on both sides of an isolation room and provided with water and pelleted feed *ad libitum.* The floor area of this room was 33.1 m^2, and the air was replaced approximately 12 times/hr (similar to the ventilation rate at mild temperatures in commercial poultry houses). The floor was cleaned at weekly intervals to remove accumulated waste and debris (including manure, feathers, and dust). All hens were inoculated orally with a phage type 13a isolate of *S. enteritidis* (10,11) prepared by overnight incubation at 37 C in tryptone soya broth (Oxoid Limited, Basingstoke, Hampshire, U.K.) and dilution in 0.85% saline to yield approximately 1.2×10^9 colony-forming units of *S. enteritidis* per 1-ml dose.

Fecal samples. Samples of voided feces were collected from each hen and cultured for the presence of *S. enteritidis* by previously described methods (20) immediately before inoculation and at 1, 2, 3, and 4 wk postinoculation.

Detection of specific antibodies. Yolks from eggs collected immediately before inoculation and at weekly intervals after inoculation were tested for the presence of antibodies specific to *S. enteritidis* flagella by an enzyme-linked immunosorbent assay (ELISA) developed by Holt and Porter (26) and described previously (17). Postinoculation egg yolk samples were considered to be antibody positive in this test if their ELISA absorbance values exceeded the mean absorbance value for the preinoculation negative control samples by more than two standard deviations.

Air samples. To test for the presence of airborne bacteria in the room containing infected laying hens, plates of agar media were exposed to air samples collected by three methods. All air samples were collected by placing the necessary apparatus and media on top of the upper tier of cages, near the middle of the row on each side of the room. The first air sampling method simply involved passive exposure of uncovered plates of agar media for periods of 20 min, 1 hr, or 3 hr. Four plates were exposed each time this test was performed. The second air sampling method employed an SAS Super 90 Impaction Air Sampler (Bioscience International, Rockville, MD) that directed air onto the surface of agar media plates at a rate of 90 liters/min. One plate was exposed each time this test was performed. Impaction samples were collected only for 20-min intervals because longer exposure overloaded the agar plates with too many bacterial colonies to allow enumeration or identification.

The third air sampling method applied an experimental electrostatic sampling device, developed and produced at our laboratory. This device is simple (with no moving parts), compact (0.9 kg; 20 cm long $\times$ 14 cm wide $\times$ 15 cm high), battery operated (up to 5 hr on a single 9 V battery), and inexpensive (built with $50 of parts). All electronic components except for the plug-in batteries are housed in a completely waterproof enclosure to allow disinfection by spraying after use. The device operates by applying a strong electrostatic field to draw charged airborne dust particles and aerosols onto the surface of agar media plates. As charged particles are drawn to the media, adjacent air is pulled in behind them—similar to the effect of air being pulled down with rain as it falls from a storm cloud—and the charged particles and associated airborne microorganisms are tightly bound to the media by electrostatic attraction. The electrostatic sampling device (with two plates each time) was used to collect air samples for 20 min, 1 hr, or 3 hr.

Air samples were collected and tested four times each week for 4 wk postinoculation. On 2 days each week, air samples were collected onto brilliant green (BG) agar (Becton Dickinson, Franklin Lakes, NJ) supplemented with 0.02 mg/ml of novobiocin (Sigma Chemical Co., St. Louis, MO). This medium is highly selective and differential for salmonellae. On the other 2 days, air samples were tested by exposure of plates of MacConkey (MAC) agar (Becton Dickinson). This medium supports the multiplication of a wide range of enteric bacteria, including salmonellae and coliforms. MAC agar samples were collected only at exposure intervals of 20 min and 1 hr to minimize bacterial overgrowth. Samples with the two types of media were taken on different days to accommodate scheduled visits to the containment building by animal caretakers. Air samples were also taken (on BG agar only) on the day before inoculation of hens with *S. enteritidis*.

After exposure during sampling, the agar plates were incubated for 24 hr (MAC agar plates) or 40 hr (BG agar) at 37 C. The total number of bacterial colonies on each MAC agar plate and the number of typical *Salmonella* colonies on each BG agar plate were determined and recorded after incubation. Biochemical and serologic confirmation by standard methods (38) was used to verify the presumptive visual identification of typical *S. enteritidis* colonies on all BG agar plates. No other *Salmonella* serotype was detected in this study.

Statistical analysis. For each replicate trial, significant differences ($P < 0.05$) between air sampling methods in the mean number of total or *Salmonella* colonies recovered on agar media plates were determined by Kruskal–Wallis analysis of variance followed by Dunn multiple comparison test. Data were analyzed with Instat biostatistics software (GraphPad Software, San Diego, CA). Because the statistical relationships between treatment groups were similar for the two trials, the results were combined for analysis.

RESULTS

Oral inoculation with *S. enteritidis* established intestinal colonization (measured by testing for fecal shedding) and led to invasive infection (measured by

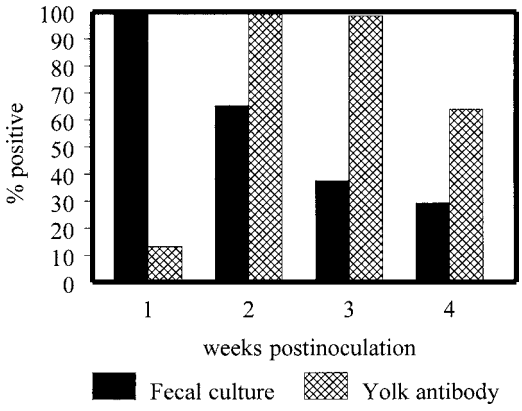

Fig. 1. Frequency of recovery of *Salmonella enteritidis* from samples of voided feces and frequency of positive *S. enteritidis*–specific egg yolk antibody assay results after oral inoculation of laying hens. Samples were obtained from 36 hens in each of two replicate trials.

testing for specific egg yolk antibodies) in the hens. No fecal samples collected before inoculation were positive for *Salmonella*, but all such samples were positive for *S. enteritidis* at 1 wk after inoculation (Fig. 1). The frequency of fecal shedding of *S. enteritidis* then declined steadily to 29.2% at 4 wk postinoculation. Similarly, all tested hens yielded positive ELISA results for *S. enteritidis*-specific egg yolk antibodies by 2 wk postinoculation (Fig. 1). The frequency of egg yolk antibody detection decreased to 63.8% of the hens by 4 wk postinoculation.

All three methods for sampling air from rooms containing infected chickens led to the collection of bacterial colonies on MAC agar plates (Table 1). The mean total bacterial counts for all combinations of air sampling method and duration increased to peak values at 3 wk postinoculation and then declined at 4 wk postinoculation. The electrostatic sampling device was significantly ($P < 0.05$) more efficient than passive exposure in attracting bacteria to MAC agar plates on each sampling date. Moreover, the impaction sampler never yielded significantly higher total bacterial recovery than did the electrostatic device at any of the four postinoculation intervals. No other combination of air sampling method and duration resulted in more total bacterial colonies on MAC agar (on any sampling date) than were obtained by applying the electrostatic device for 1 hr. This method produced an overall mean (for all 4 wk combined) of 66.6 bacterial colonies per MAC agar plate, but all other sampling approaches led to the recovery of 22.8 or fewer colonies.

All three air sampling methods also supported the collection of typical *Salmonella* colonies on BG agar plates (Table 2). All preinoculation air samples were negative for *Salmonella*. At 1 wk after oral inoculation of the hens with *S. enteritidis*, at least 87.5% of the agar plates were positive for *Salmonella* after testing with either the impaction sampler or electrostatic device for 20 min or after testing by passive exposure for 3 hr. The frequency of positive results declined in subsequent weeks for all methods, but both the impaction sampler (used for 20 min) and the electrostatic device (used for 1 or 3 hr) recovered *Salmonella* from at least 37.5% of air samples taken at 4 wk postinoculation. For all four sampling dates combined, no other combination of air sampling method and duration yielded more frequent isolation of *Salmonella* colonies on BG agar than was obtained with the electrostatic device for

Table 1. Bacterial colonies on MacConkey agar plates exposed to various types of air samples in rooms containing laying hens inoculated with *Salmonella enteritidis*.[A]

Duration of sampling	Sample type[B]	Mean total number of bacterial colonies				
		1 wk PI[C]	2 wk PI	3 wk PI	4 wk PI	All wks
20 min	Impaction	21.0^{ab}	23.0^{ab}	39.5^{ab}	7.5^{ab}	22.8^{ab}
	Electrostatic	12.8^{ab}	22.5^{a}	29.5^{ac}	9.3^{a}	18.5^{ab}
	Passive	3.3^{a}	1.4^{b}	4.4^{ad}	0.1^{b}	2.3^{c}
1 hr	Electrostatic	68.5^{b}	83.3^{a}	94.0^{bd}	20.5^{a}	66.6^{a}
	Passive	13.1^{ab}	6.9^{ab}	55.6^{bc}	2.3^{ab}	19.5^{b}

[A]In each of two trials, 36 orally infected laying hens were housed in individual cages. Values within columns are significantly different ($P < 0.05$) if they share no common lowercase superscript.

[B]Collected with an impaction sampler (two plates for each sampling duration) or an electrostatic sampling device (four plates each time) or by passive exposure to air (eight plates each time).

[C]PI = postinoculation.

Table 2. Typical *Salmonella* colonies on brilliant green agar plates exposed to various types of air samples in rooms containing laying hens inoculated with *Salmonella enteritidis*.[A]

Duration of sampling	Sample type[B]	Mean number of *Salmonella* colonies (positive plates/total)				
		1 wk PI[C]	2 wk PI	3 wk PI	4 wk PI	All wks
20 min	Impaction	13.0^{ab} (4/4)	1.0^{ab} (2/4)	0^{a} (0/4)	0.8^{a} (2/4)	3.7^{ab} (8/16)
	Electrostatic	6.4^{ab} (7/8)	0.1^{a} (1/8)	0.5^{a} (3/8)	0.3^{a} (1/8)	1.8^{ac} (12/32)
	Passive	0.8^{a} (7/16)	0.1^{a} (2/16)	0^{a} (0/16)	0.1^{a} (1/16)	0.2^{a} (10/64)
1 hr	Electrostatic	3.5^{bc} (8/8)	1.4^{ab} (5/8)	1.4^{a} (4/8)	0.6^{a} (3/8)	1.7^{bc} (20/32)
	Passive	0.5^{a} (5/16)	0.1^{a} (2/16)	0.3^{a} (3/16)	0.2^{a} (3/16)	0.3^{a} (13/64)
3 hr	Electrostatic	8.6^{b} (8/8)	2.8^{b} (8/8)	4.6^{a} (4/8)	2.3^{a} (4/8)	4.6^{b} (24/32)
	Passive	1.8^{ac} (14/16)	0.2^{a} (2/16)	1.6^{a} (7/16)	0.3^{a} (4/16)	1.0^{ac} (27/64)

[A]In each of two trials, 36 orally infected laying hens were housed in individual cages. Values within columns are significantly different ($P < 0.05$) if they share no common lowercase superscript.
[B]Collected with an impaction sampler or an electrostatic sampling device or by passive exposure to air.
[C]PI = postinoculation.

1 hr (62.5% of plates) or 3 hr (75% of plates). During the first 2 wk postinoculation (and for all 4 wk combined), the electrostatic sampling device (used for 1 or 3 hr) was associated with significantly ($P < 0.05$) greater mean numbers of *Salmonella* colonies on BG agar plates than were obtained by passive exposure. No significant differences in the mean numbers of *Salmonella* colonies were evident between the impaction sampler and the electrostatic device. However, only the electrostatic sampling device (used for 3 hr) collected a mean of at least two typical *Salmonella* colonies per agar plate on each of the four sampling dates.

DISCUSSION

The present study determined that several sampling methods were able to detect airborne *S. enteritidis* in a room containing infected laying hens for up to 4 wk after oral inoculation. Air sampling has not been a common feature of *Salmonella* testing programs for commercial poultry, but experimental documentation of the airborne transmission of *S. enteritidis* infection (15,24,30,33) suggests that this pathogen is likely to be found circulating in the air of infected flocks or contaminated houses. However, available data from prior studies to evaluate the various potential sampling methods for detecting airborne bacteria in poultry houses are very limited in both number and scope. In a pair of experiments, bacteriologic culturing of air filters from poultry housing facilities was less sensitive for detecting *Salmonella* than was culturing of floor litter (28), but air filters were superior to litter samples for use with a gene amplification method (29). Impaction samplers have been applied successfully to recover airborne salmonellae in turkey rearing houses (27), and passive exposure of agar plates was used to recover airborne salmonellae in broiler chicken hatching cabinets (2). In a comparative study, passive exposure of agar plates was more sensitive than impaction sampling for detecting *Salmonella* in air samples from a broiler hatching cabinet (4).

In the present study, the electrostatic sampling device consistently achieved significantly better detection of both total bacteria on MAC agar and *S. enteritidis* on BG agar than was obtained by passive exposure of agar plates. The recovery of salmonellae by passive exposure was not improved by extending the duration of sampling from 3 to 24 hr (data not shown). Although statistically meaningful comparisons between the electrostatic and impaction samplers were made difficult by the small number of impaction samples collected, the impaction sampler was not demonstrably superior to the electrostatic device for recovering either total bacterial colonies on MAC agar or *Salmonella* colonies on BG agar. Passive exposure of agar plates is the most commonly used method for detecting airborne bacterial pathogens, but this approach is relatively insensitive unless very lengthy exposure intervals are used. The portable, medium-volume impaction sampler used in this study cost approximately 100 times more than the electrostatic device. Higher volume air samplers can cost even more and are typically large and difficult to disinfect. The electrostatic sampling device is small, inexpensive, and easily disinfected. Overall, the electrostatic device, used for a 3-hr sampling interval, provided the most dependable

detection of airborne *Salmonella* by any method considered in this study.

The probability of recovering airborne bacteria may depend on both the sensitivity of the selected testing method and the concentration of bacterial cells circulating in the air at the sample collection site. The number and location of air samples necessary for consistent detection of *S. enteritidis* in a poultry house of commercial size and scale are not yet known. The previously documented ability of *S. enteritidis* to persist in poultry houses for many months (6,7) should result in a correspondingly persistent presence of the pathogen in association with airborne dust and aerosols. In the present study, the number of *S. enteritidis* cells recovered by air sampling declined steeply after the first week postinoculation, although all three sampling methods still yielded positive results at 4 wk postinoculation. This decline was probably influenced by both the weekly removal of accumulated sources of dust when the floor was cleaned and the steadily dropping frequency of fecal shedding of *S. enteritidis* into the environment by infected hens. Both fecal shedding and specific antibody production have previously been shown to be useful for predicting the likelihood that a flock will lays eggs contaminated by *S. enteritidis* (14,19). Moreover, the presence of *S. enteritidis* in the laying house environment of commercial flocks has been strongly correlated with the production of contaminated eggs (22,23). The predictive relevance of detecting airborne *S. enteritidis* has not been established but would likely be similar to that of other parameters of environmental contamination.

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